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Effects of Salinity on Sperm Motility, Fertilization, and Development in the Pacific Herring, *Clupea pallasi*

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Abstract. We investigated the effects of salinity on fertilization and early development in a population of Pacific herring, *Clupea pallasi*, that migrate from oceanic waters into the San Francisco Bay estuary to spawn. The salinity range for fertilization fell between 8 and 28 ppt, with an optimal range of about 12 to 24 ppt. In comparison, the range for a population of *C. harengus membras* (Airisto Sound, Finland) that reside year-round in the Baltic Sea was 4 to 24 ppt. Roles for both Na^+ and K^+ were indicated in *C. pallasi* fertilization since increasing Na^+ in the presence of 10 m*M* K^+ (concentration of seawater) mimicked the effects of increased overall salinity, whereas reduced effects were obtained if $[K^+]$ was held at 5 m*M* (that of half-strength seawater). The initiation of *C. pallasi* sperm motility by components of the egg chorion, a prerequisite for fertilization, was inhibited at both elevated (28 and 32 ppt) and reduced (4 and 8 ppt) salinities. Embryonic development through larval hatching in *C. pallasi* exhibited a salinity tolerance similar to that of fertilization; optimum development was obtained at salinities between 8 and 24 ppt. A comparison of developmental progression in 3.5, 14, and 28 ppt seawater revealed that salinity effects became evident during the post-gastrulation stages of development and that progression to hatching was delayed in both the lower and higher salinities for those embryos that completed development.

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Abbreviations: ASW, artificial seawater; FSW, filtered seawater; HR, herring Ringer's; SMIF, sperm motility initiation factor.

Introduction

Pacific herring, *Clupea pallasi*, inhabit the continental shelves of the North Pacific Ocean from California to Korea, and the White and Kara Sea regions of the Arctic Ocean (Dushkina, 1973; Grant, 1984; Grant and Utter, 1984). Once considered a subspecies of the Atlantic herring (*C. harengus*), as currently is the Baltic herring, *C. pallasi* is now recognized to be different behaviorally, morphologically, and genetically from *C. harengus* (Grant, 1984; Grant and Utter, 1984; Haegele and Schweigert, 1985). Pacific herring spawn during the winter and spring, predominantly in protected waters of bays and estuaries. Males release sperm into the water while females deposit adhesive eggs onto shallow subtidal and lower intertidal substrates where fertilization and embryonic development take place (see Haegele and Schweigert, 1985). Herring are unique among fish in that sperm are immotile when released from males and remain so after dilution into the surrounding media (Yanagimachi and Kanoh, 1953). They are typical in that their eggs are surrounded by a thick chorion with only one access site to the egg surface (the micropylar canal), and that sperm must be motile to traverse this canal and achieve fertilization (Yanagimachi and Kanoh, 1953; Yanagimachi, 1956; 1957).

Although salinity has been shown to influence fertiliza-

tion and embryonic development in *C. pallasi*, the importance of this environmental factor has been questioned due primarily to the overall broad range of salinities (5–35 ppt) observed at spawning sites throughout the species' range (Blaxter and Holliday, 1963; Haegele and Schweigert, 1985). Laboratory studies, however, with specific populations or stocks suggest that this broad tolerance range may not apply to all Pacific herring. An optimum laboratory salinity range of 10–36 ppt has been reported for the embryonic development of White Sea herring (Dushkina, 1973), and herring from the Strait of Georgia in British Columbia have shown a developmental tolerance of 12–26 ppt (Alderdice and Velsen, 1971) with a much narrowed optimum of 12–17 ppt (reviewed by Alderdice and Hourston, 1985).

Research into the mechanisms of fertilization in *C. pallasi* has provided insight as to how salinity could adversely affect the establishment of a new generation. Herring sperm become motile upon physical contact with the micropyle region of the egg chorion, and both K^+ and Na^+ ions have been implicated in this initiation of sperm motility (Yanagimachi and Kanoh, 1953; Yanagimachi, 1956; 1957; Yanagimachi *et al.*, 1992). The presence of extracellular K^+ is required for initiation of motility at the micropyle region of an egg (Yanagimachi *et al.*, 1992). In contrast, Na^+ is not required and, in fact, the deletion of Na^+ or the addition of a Na^+ ionophore (in low external Na^+ concentrations) initiates spontaneous sperm motility in the absence of the chorion factor (Yanagimachi *et al.*, 1992).

In the present report we (1) describe the salinity tolerance range for fertilization in *C. pallasi* from San Francisco Bay, California; (2) provide evidence that a synergistic effect between elevated Na^+ and K^+ ions at elevated salinities is responsible for decreased fertilization in *C. pallasi;* (3) demonstrate that decreased fertilization at elevated salinities is due to an inhibition of sperm motility; and (4) determine the effects of lowered and elevated salinity on embryonic development in *C. pallasi.* Lastly, we compare the salinity tolerance range for fertilization of an eastern population of Baltic herring (*C. h. membras*) with that of San Francisco Bay *C. pallasi.*

Materials and Methods

Collection of animals and gametes

Pacific herring, *Clupea pallasi,* were collected by otter trawl from the San Francisco Bay estuary in collaboration with biologists from the California Department of Fish and Game. The fish were maintained at 4°C in moist conditions and brought to the University of California Bodega Marine Laboratory within 3–4 h of collection. Eggs were obtained either by stripping or by dissection of ovaries as described previously (Yanagimachi, 1957; Griffin *et al.*, 1996). "Dry" eggs, in ovarian fluid, were maintained in a humid chamber at 4°C until used. Testes were dissected from males and maintained separately in a moist environment at 4°C until used (Yanagimachi *et al.*, 1992). To obtain sperm, milt was collected by stripping or by dissection of testes, diluted to approximately 10^9 sperm/ml into herring Ringer's (HR), and kept at 4°C: this maintains sperm in an isotonic medium and in an inactivated state (Yanagimachi *et al.*, 1992). HR was prepared according to Yanagimachi (1957) and contained 206 m*M* NaCl, 7.2 m*M* KCl, 2.1 m*M* $CaCl_2$, 3.3 m*M* $MgCl_2 \cdot 6H_2O$, pH adjusted to 7.8 using 1 m*M* $NaHCO_3$. Sperm were used for experiments within 1 h after isolation into HR.

Baltic herring (*C. h. membras*) from Airisto Sound (Turku, Finland) were collected in trapping pens at spawning sites within Airisto Sound, the gonads dissected, and then shipped overnight under moist conditions, at 4°C, to the Bodega Marine Laboratory. Gametes were isolated as described for *C. pallasi.*

Fertilization assays under different salinity conditions

Fertilization curves based on sperm concentration were generated for each set of gametes to determine the lowest sperm concentration that yielded between 50% and 90% fertilization. This was done to ensure that effects of experimental manipulations were not masked by an excess of sperm. For *C. pallasi,* the ratio was determined in half-strength filtered (0.45-μm) seawater ($^1/_2$ FSW), pH 7.8, previously shown to be optimal for fertilization in this species (Yanagimachi *et al.*, 1992). For *C. h. membras,* $^1/_4$-strength FSW was utilized, based on the reported salinities of 6–7 ppt in Airisto Sound, Finland (Vuorinen and Ranta, 1987). Different salinities of seawater were prepared by adding appropriate quantities of double-distilled water to full-strength FSW, after which salinity in parts per thousand (ppt) was determined with a refractometer. Since the salinity tolerance range is reported to expand close to optimal temperatures and to contract as temperature increases or decreases away from the optimum (Alderdice and Velsen, 1971), the effects of temperature were eliminated by conducting experiments within normative environmental temperatures (Alderdice and Velsen, 1971; Haegele and Schweigert, 1985). Unaltered salinities of FSW at the facility during fertilization experiments ranged from 32 to 34 ppt; if the salinity was above 32 ppt, distilled water was used to bring it down to 32 ppt. For all fertilization experiments, stock sperm (in HR) were diluted into 10 ml of the appropriate dilution of FSW (in polystyrene culture dishes) and incubated for 10 min at 12°C, after which about 100 eggs were distributed evenly into the dishes. Eggs were co-incubated with sperm for 10 min, quickly rinsed twice with the same-

salinity FSW to remove excess sperm, and incubated at 12°C, 10 h light/14 h dark, in temperature- and light-controlled incubators (Revco and Lab-Line Ambi-Hi-Lo). For each salinity treatment, fertilization was determined by scoring the percentage of eggs with elevated chorions at 30 min post-insemination (see Fig. 1); these zygotes were followed through first cleavage to ensure that they had been fertilized. Experiments were conducted in triplicate ($n = 3$) using gametes from different animals; each n was, in turn, the average of three replicates using gametes from the same animals. Data presented are the averages ± standard deviations of the replicates (n). Significance of differences was determined with a Student's t test using the values from each n.

Effects of extracellular Na^+ and K^+ concentration on fertilization

Artificial seawaters containing different concentrations of Na^+ and K^+ were prepared from half-strength ASW (artificial seawater; from Cavanaugh, 1975) containing either 5 m*M* or 10 m*M* K^+. To obtain final Na^+ concentrations of 200–400 m*M*, the ½ ASWs were supplemented with Na^+ in 50-m*M* increments using a stock solution of 4.4 *M* NaCl. Fertilization assays in these media were conducted as described above.

In vitro *sperm motility assessments under different salinity conditions*

Sperm motility was assessed *in vitro* using Pacific herring sperm motility initiation factor (SMIF) according to Yanagimachi *et al.* (1992) and Pillai *et al.* (1993).

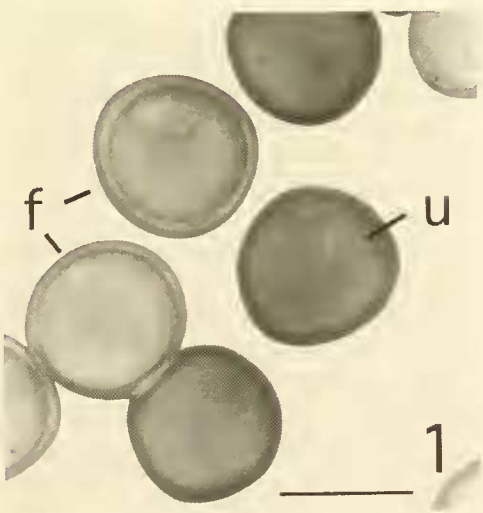

Figure 1. Fertilized (f) and unfertilized (u) eggs of *Clupea pallasi*. Fertilized eggs are translucent with an easily seen perivitelline space between chorion and egg. Bar = 1 mm.

Chorions from *C. pallasi* eggs were obtained as described by Griffin *et al.* (1996), and SMIF was isolated from these chorions according to Pillai *et al.* (1993). Different-salinity FSWs were mixed with SMIF (at 50–100 μg protein SMIF/ml, final concentration), and 50 μl of each was placed into individual wells of a multi-well immunoslide (Polysciences, Inc., PA). Stock sperm (10^9 sperm/ml in HR) diluted (final concentration of 5×10^6 sperm/ml) into different-salinity FSWs were then added to the wells containing SMIF (in the same-salinity FSWs) as a 1.5-μl bolus. Using darkfield microscopy and a 20× objective lens, the interface between the bolus of sperm and the SMIF solution was monitored for 15 s. In the absence of motility the bolus spread into the SMIF as a monolayer of sperm, preserving a discrete interface between the two. The initiation of motility, characterized by a counterclockwise corkscrew or circular swimming pattern, disrupted this interface; the degree of disruption was related to the percentage of motile sperm. Motility was scored by using the following qualitative motility index: 0 = no motility, 1 = up to 25% of the cells motile, 2 = up to 50% of the cells motile, 3 = up to 75% of the cells motile, 4 = >75% of the cells motile (Griffin *et al.*, 1996).

Embryonic development and hatching of C. pallasi

For assessing the effects of altered salinities on embryonic development and hatching, *C. pallasi* eggs were fertilized in ½ FSW and then cultured in different-salinity seawaters as described above. During the embryonic development experiments, unaltered salinities ranged from 28–32 ppt. When the full-strength salinity was below 32 ppt, the salinity was not adjusted upward, but the lower starting salinity was noted. The FSW of the cultures was exchanged every 24 h and salinities, dissolved oxygen, and pH were measured at the end of each experiment. Any experiment that exhibited significantly decreased dissolved oxygen or altered pH was not included in data analyses. Development experiments were conducted on batches of embryos that were different from those used for the fertilization experiments described above.

Embryonic development and hatching were followed using either an Olympus stereo-zoom microscope equipped with darkfield optics or an Olympus BHS compound microscope equipped with Nomarski optics. Development was assessed on the basis of comparisons with control embryos and using terminology and stages employed by Kimmel *et al.* (1995) for the zebrafish (see Results). Larval hatching was quantified by counting empty chorions still attached to the bottom of the dishes *versus* chorions containing unhatched embryos. In the experiments from which the salinity tolerance curve was generated, hatching success in all salinities was assessed when greater than 40% of the embryos in the ½ FSW had

hatched. In the experiments to ascertain whether salinity delayed hatching, the cultures were scored at 24-h intervals for 3 days after hatching reached 40% in ½ FSW cultures. In these experiments the salinity of FSW was 28 ppt; therefore, the different-salinity FSWs were 3.5 ppt (⅛ FSW), 14 ppt (½ FSW), and 28 ppt (full-strength FSW). The total number of chorions was counted each day to determine if embryos or empty chorions had been inadvertently removed during water exchanges in cultures.

Results

The salinity tolerance ranges for fertilization in *C. pallasi* from San Francisco Bay, California, USA, and *C. h. membras* from Airisto Sound, Turku, Finland, overlap, but are significantly different (Figs. 2, 3). The optimum salinity for *C. h. membras* in our experiments was 8 ppt (56.3% ± 12.7% fertilization), with decreases in fertilization to 15.0% ± 2.2% at 4 ppt and 10.5% ± 3.8% at 24 ppt (Fig. 2). Fertilization in *C. pallasi* peaked at 16 ppt (86.0% ± 7.5%) with decreases to 17.1% ± 4.6% at 8 ppt and 10.5% ± 6.2% at 32 ppt. After normalization of the data such that the highest fertilization percentage observed for each species was designated as 100% (Fig. 3), the significance of the differences in salinity tolerance was determined. In 8 ppt, fertilization of *C. h. membras* was significantly higher ($P < 0.001$) than that of *C. pallasi* (see Fig. 3). At 24 ppt, the difference between the two species was also significant ($P < 0.01$), but reversed: fertilization was 71.1% ± 12.9% in *C. pallasi*, but 20.3% ± 13% in *C. h. membras*. Within the mid-range, 16 ppt, there was also a difference in fertilization ($P < 0.05$): 100% for *C. pallasi* and 78.6% ± 12.5% for *C. h. membras*. The salinity response curves for *C. pallasi* and *C. h. membras* were very similar, except that the *C. pallasi* curve was shifted 4–8 ppt hypersaline to the one for *C. h. membras*. Thus, although each species exhibited an optimal range of nearly 20 ppt, the position of that range differed between *C. h. membras* and *C. pallasi*.

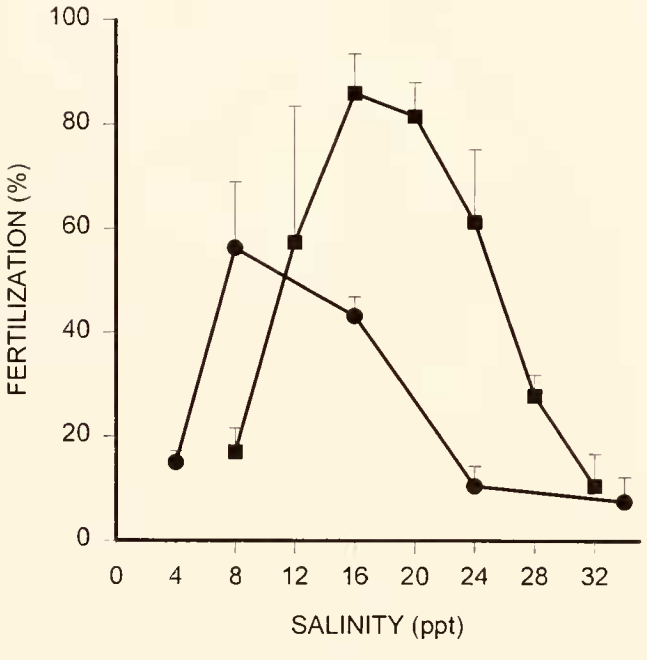

Figure 2. Fertilization in *Clupea pallasi* from San Francisco Bay (■) and *C. h. membras* from Airisto Sound, Finland (●) in seawater of different salinities; $n = 3$. Vertical lines = std. dev.

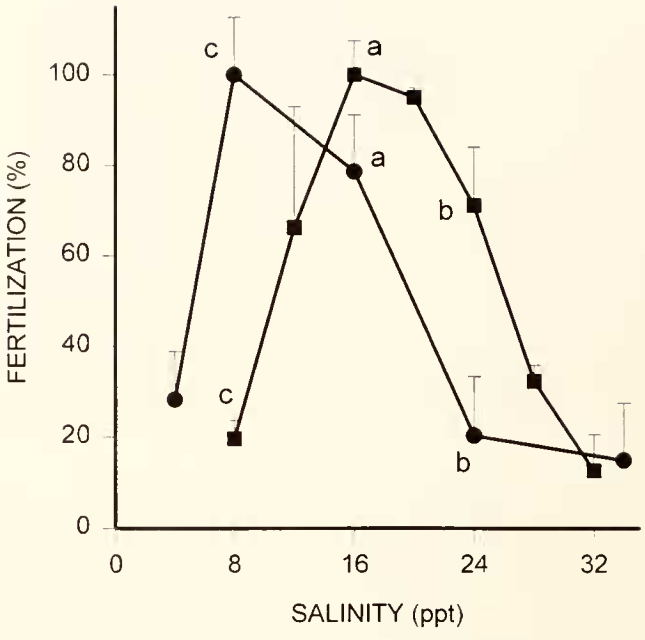

Figure 3. Normalized fertilization percentages for *Clupea pallasi* (■) and *C. h. membras* (●) at different salinities. Percentages are normalized from data in Figure 2; $n = 3$. Vertical lines = std. dev. a-a, $P < 0.05$; b-b, $P < 0.01$; c-c, $P < 0.001$ (Student's t test).

The decrease in *C. pallasi* fertilization at increased salinity was a function of the external concentrations of both Na^+ and K^+ (Fig. 4). In 211 m*M* Na^+ (concentration of ½ ASW), fertilization was similar in the presence of 5 m*M* K^+ (concentration in ½ ASW) and in 10 m*M* K^+ (concentration in full-strength seawater): 67% ± 8.7% fertilization was obtained in ½ ASW when the $[K^+]$ was 5 m*M*, and 74.7% ± 1.5% of the eggs fertilized when the $[K^+]$ was 10 m*M*. Likewise, raising the external $[Na^+]$ to 400 m*M* while keeping $[K^+]$ at 5 m*M* did not significantly change fertilization levels. Fertilization was significantly affected ($P < 0.005$) at $[Na^+]$s of 300, 350, and 400 m*M* when the $[K^+]$ was raised to 10 m*M* (Fig. 4).

Sperm behavior around the micropyles of eggs (*C. pallasi*) in both high- and low-salinity seawaters suggested that the numbers of motile sperm were reduced compared to those at optimal salinity (16 ppt). To more accurately

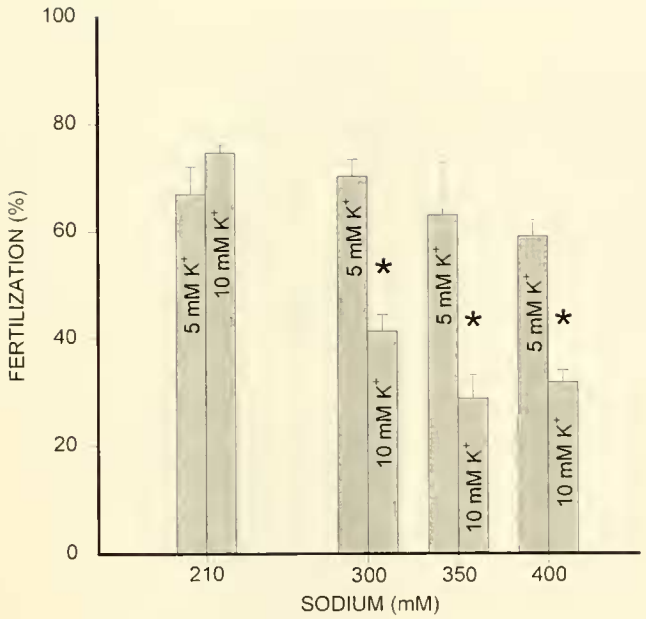

Figure 4. Effects on fertilization of increasing [Na^+] in half-strength filtered seawater containing 5 m*M* K^+ and 10 m*M* K^+; $n = 3$. Vertical lines = std. dev. Fertilization is significantly lower (*) in the 10 m*M* K^+ treatment than in the 5 m*M* K^+ treatment at the same salinity ($P < 0.01$, Student's t test).

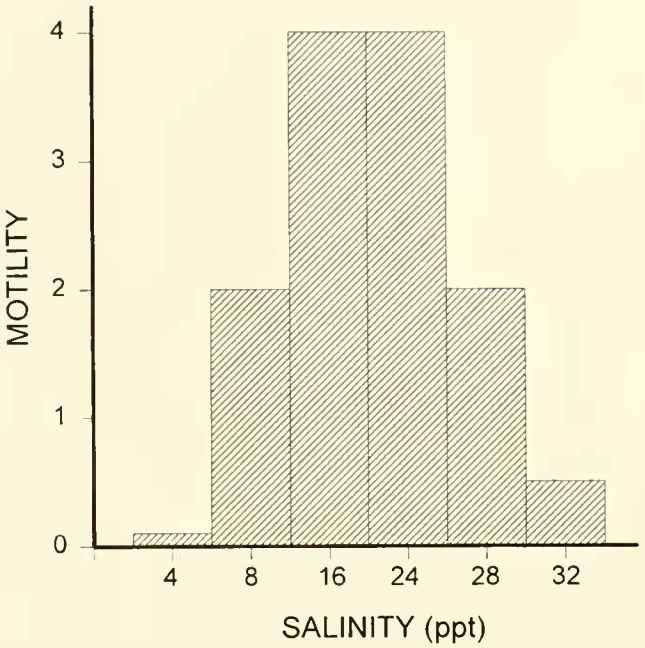

Figure 5. Sperm motility as a function of salinity. Sperm motility was induced *in vitro* with sperm motility initiation factor at the salinities listed above; $n = 3$. Motility was scored as 4 > 75% motile, 3 = 50%–75% motile, 2 = 25%–50% motile, and 1 < 25% motile.

evaluate the effects of salinity on herring sperm, motility was assayed *in vitro*, using *C. pallasi* sperm and SMIF that was isolated from *C. pallasi* eggs. When sperm were exposed to SMIF at differing salinities, motility was a function of external salinity (Fig. 5) and mirrored the range observed for fertilization at different salinities (compare Figs. 2 and 5). Motility was greatest at 16 and 24 ppt and lowest at extreme salinities of 4 and 32 ppt, although at neither extreme was motility completely inhibited.

First described by McMynn and Hoar (1953), the events and stages of embryonic development in *C. pallasi* closely follow those recently described in detail for the zebrafish by Kimmel *et al.* (1995), and so their terminology has been adopted in the current study. Five stages were scored during experiments on salinity tolerance of embryonic development. (1) First cleavage occurred at about 3 h post-insemination in ½ FSW at 12°C. (2) The onset of gastrulation occurred at about 16–17 h and corresponded with the embryo reaching 50% epiboly; gastrulation was completed by 20–21 h. (3) The 5-somite stage of segmentation was seen at 42–48 h and was marked by the appearance of optic vesicles. (4) Retinal pigmentation (entrance into the pharyngula period) was initiated by day 5 and progressed through day 6 of development. (5) Hatching of the larva occurred at 8–10 days post-fertilization in ½ ASW.

Embryonic development, like fertilization, was affected by external salinity. When measured as a function of the effect on an embryo's ability to complete development through hatching, the optimal salinities were between 8

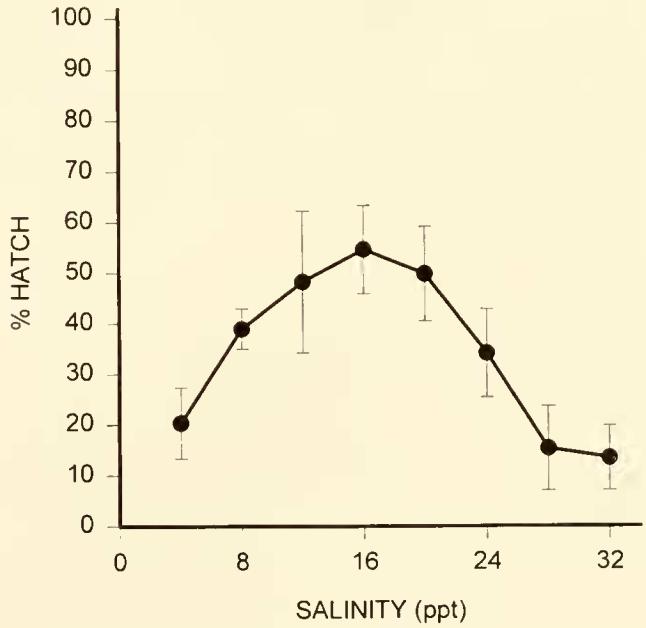

Figure 6. Effects of salinity on hatching of *Clupea pallasi* larvae as of day 9 post-fertilization; $n = 3$. Vertical lines = std. dev.

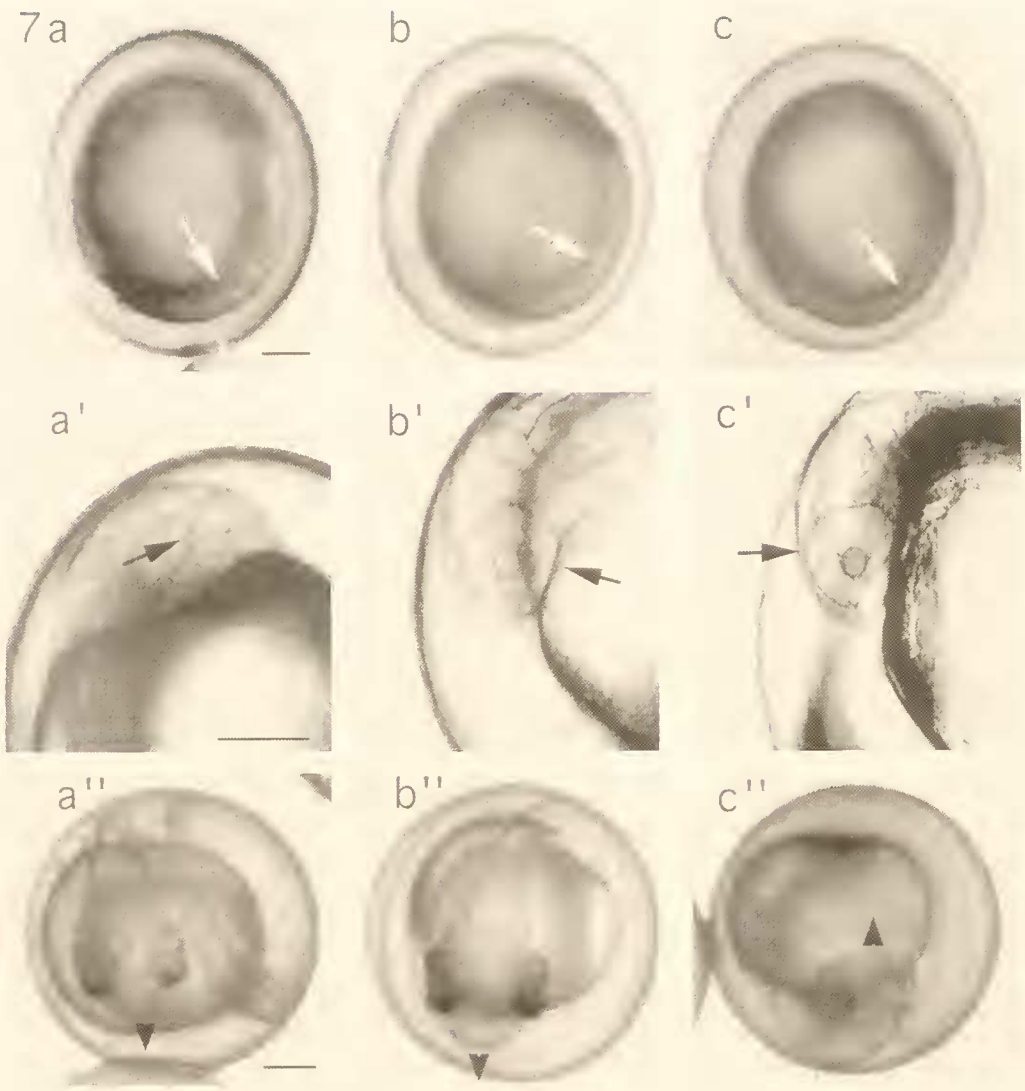

Figure 7. Comparison of development in 3.5 ppt (a-a″), 14 ppt (b-b″), and 28 ppt (c-c″) seawater. Bars = 200 μm. (a-c) Embryos at 21 h post-fertilization nearing the completion of epiboly (arrows = edge of epibolic front; arrowheads [panel b] show future head and tail regions of embryo). (a′-c′) Sixty-six hour embryos at lens stage of eye development (arrows). (a″-c″) Five-day (126-h) embryos with pigmented retinas (arrowheads indicate direction the embryonic head faces).

and 24 ppt (Fig. 6). Salinities both below and above the 8–24 ppt range hindered development; 20.3% ± 7.0% of the cultured embryos hatched in 4-ppt FSW, which was significantly less ($P < 0.01$) than the 38.9% ± 4.0% that hatched in 8-ppt FSW. Likewise, only 15.3% ± 8.3% and 13.4% ± 6.4% of embryos hatched in 28- and 32-ppt FSW, respectively, significantly lower ($P < 0.01$) than the percentage that hatched in 24-ppt FSW (34.1% ± 8.7%).

To further examine the effects of low and high salinity, the course of development of embryos was followed in three FSW salinities: 28 ppt (full-strength seawater; see Materials and Methods), 14 ppt, and 3.5 ppt. Salinity had no observable significant effect on first cleavage (not shown), epiboly, optic vesicle formation, or initiation of retinal pigmentation. The percentage of fertilized embryos that completed development through epiboly in all salinities was 74%–84%, with no significant difference between salinities (Fig. 7a–c). Near the end of day 3 (66 h), lens development was evident (Fig. 7a′-c′); by day 5, retinal pigmentation was evident in embryos at all three salinities (Fig. 7a″-c″). A salinity-associated delay in hatching and in the ability to hatch was observed (Fig. 8). At 9 days post-fertilization, 42.5% ± 4.6% of embryos in the 14-ppt cultures had hatched, whereas 17% ± 6.9% and 18.2% ± 3.4% had hatched in the 3.5- and 28-ppt

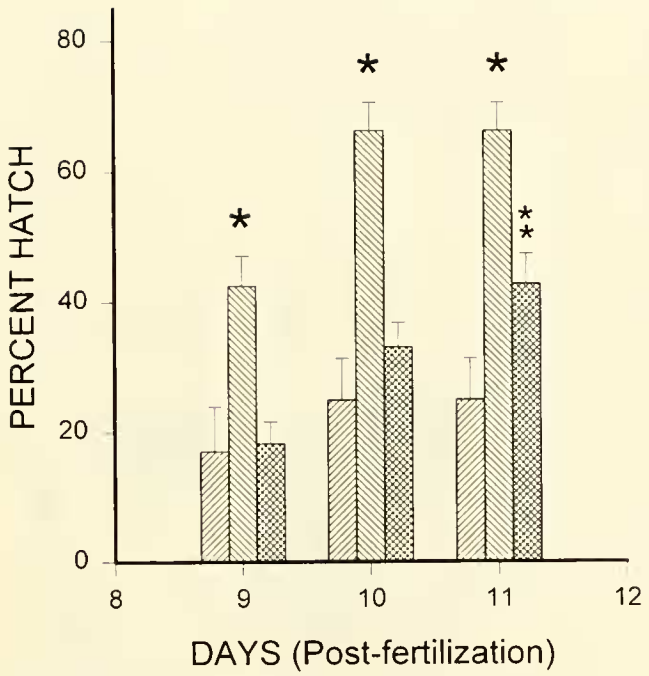

Figure 8. Effects of salinity on time to hatching and on percent hatching of *Clupea pallasi* embryos in 3.5 ppt (▨), 14 ppt (▩), and 28 ppt (□) seawater. The total percentage of hatched larvae was significantly greater (*) in 14 ppt seawater at all three days ($P < 0.001$, Student's t test). On day 11, % total hatch in the 28 ppt group was significantly higher (*) than in the 3.5 ppt group ($P < 0.005$, Student's t test).

FSWs. At day 10 the percentages increased to 24.9% ± 6.4% (3.5 ppt), 66.3% ± 4.3% (14 ppt), and 33% ± 3.8% (28 ppt). No further hatching was observed in either the 3.5- or 14-ppt cultures; however, hatching continued at 28 ppt, and by the 11th day, 42.8% ± 4.7% of the embryos had hatched. No further hatching was observed in any of the cultures. These data suggest that salinity does influence time to hatching, and thus the effect of salinity on development is not as absolute as a simple salinity tolerance curve would predict (see Fig. 6).

In addition to the delay in hatching time, both abnormal hatching and hatched abnormal embryos were observed. Figure 9 depicts larval cultures captured by video at day 10. Larvae in 14-ppt FSW swam vigorously, with few possessing abnormalities (Fig. 9a). In the other two salinities a number of abnormalities occurred, including immotile hatched larvae (see Fig. 9b), scoliotic hatched larvae (see Fig. 9c), and larvae that initiated but failed to complete hatching (see Fig. 9c). The larvae that initiated hatching but died prior to its completion were seen only in 3.5-ppt FSW cultures; by the 10th day, 30.3% ± 7.4% of the embryos had initiated, but failed to complete, the process. Again, these embryos did not survive. Scoliotic larvae varied in the degree of deformity, but none swam normally and none survived. Percentages of scoliotic larvae in both 3.5- and 28-ppt cultures averaged about 10%, whereas only 1%–2% were scoliotic at 14 ppt.

Discussion

Freshwater input has been implicated as an important component of some locations where *C. pallasi* is known to spawn (Taylor, 1971; Barnhart, 1988), and low-salinity water can stimulate spawning in the field (Rabin and Barnhart, 1986) as well as in the laboratory (Outram, 1951). There is also the suggestion in the literature that a spawning site, and therefore the salinity at which spawning occurs, may be a function of the reproductive stock (population) from which the fish derive; although some populations spawn at sites of lowered salinity, others consistently spawn where the salinity approaches that of normal, or full-strength, seawater (Dushkina, 1973; Kijima *et al.*, 1992; Kobayashi, 1993). Such isolation has been suggested to occur not only between populations that reproduce in widely separated geographical regions (Kobayashi, 1993), but also within geographical zones. Oceanic and estuarine populations of *C. pallasi* have been identified in the waters around Japan (Kijima *et al.*, 1992; Kobayashi, 1993), and populations that spawn at different salinity sites have been delineated within the White Sea, Barents Sea, Sea of Okhotsk, and Sea of Japan (Dushkina, 1973). The reproductive "stock" (population) concept is well accepted, even within regions where salinities are not known to vary widely from site to site; within British Columbia waters of the northeastern Pacific Ocean there are up to 23 reproductive stocks that are each faithful to defined spawning subregions (Hay *et al.*, 1984; Hay, 1985). What is undocumented for *C. pallasi* is whether salinity differences between sites, where they exist, lie within a broad permissive range for this euryhaline species or reflect the salinity tolerance limitations on fertilization and embryonic development in specific populations.

The results in the present study indicate that the salinity tolerances for fertilization and embryonic development in the San Francisco Bay population of Pacific herring are limited in comparison with those reported overall for the species. Furthermore, the present results both agree and disagree with previous findings. The range (between 12 and 24 ppt) at which both fertilization and development were optimal was similar to that reported for British Columbia herring (12–26 ppt with 12–17 ppt optimum) by Alderdice and Velsen (1971) and Alderdice and Hourston (1985), but more stringent than that reported by McMynn and Hoar (1953) for development of British Columbia Pacific herring (6–34 ppt) or by Dushkina (1973) for Pacific herring in the White Sea (10–36 ppt).

These differences might reflect actual abilities of populations to tolerate different salinities. Similar population

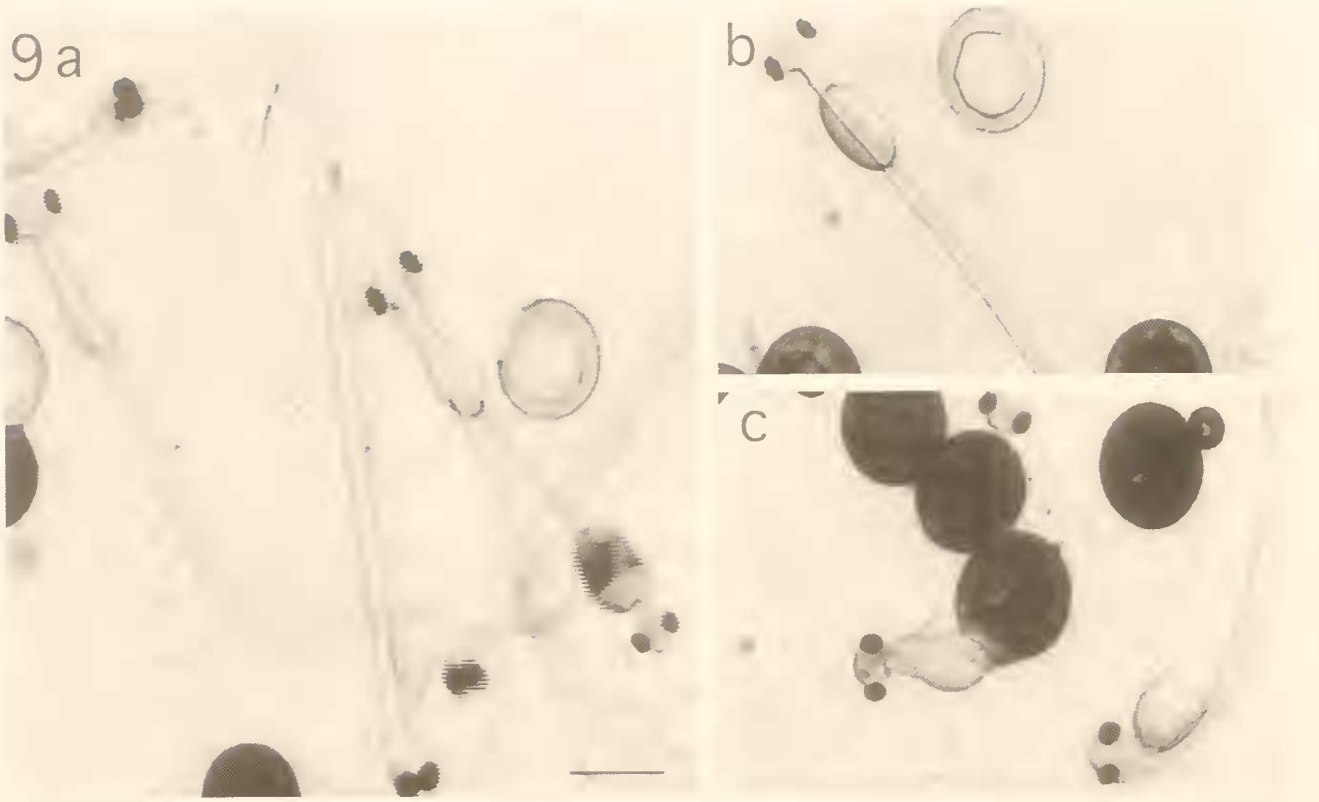

Figure 9. Representative images, obtained by video frame capture, from cultures of *Clupea pallasi* embryos maintained at three salinities. In the 14 ppt (a) and 28 ppt (b) cultures, larvae, empty (transparent) chorions of hatched larvae, and chorions (darkened, opaque) from which embryos have not hatched are evident. In the 3.5 ppt culture (c), a hatched scoliotic larva with a bent tail, a partially hatched larva, and chorions of unhatched embryos are present. Bar = 1 mm.

or subspecies differences are reported to exist within *C. harengus*, which overall possesses a broader fertilization and developmental salinity tolerance range than *C. pallasi* (see Blaxter and Holliday, 1963; Ojaveer, 1981; Haegele and Schweigert, 1985). The comparison of salinity tolerance curves for fertilization in San Francisco Bay *C. pallasi* and *C. h. membras* from Airisto Sound supports this possibility. Alternatively, the differences may reflect physiological, nongenetic responses to the different salinity regimes to which the fish are exposed. Pacific herring that reproduce in the San Francisco Bay estuary constitute the southernmost eastern Pacific population of *C. pallasi*, isolated by a large geographical distance from other Pacific populations (Haegele and Schweigert, 1985). The fish migrate from oceanic waters of the eastern Pacific into the San Francisco Bay estuary where salinities fluctuate both seasonally and annually (2–32 ppt), dependent on both natural precipitation and freshwater diversions imposed by humans (Peterson *et al.*, 1989). In contrast, the *C. h. membras* population that reproduces in Airisto Sound resides in lowered salinity waters (4–10 ppt) of the Baltic Sea throughout the year (Haapala and Alenius, 1994). The fact that the salinity tolerance range for fertilization in *C. h. membras* has not narrowed suggests that the position of the range is more responsive to the salinity history of the population than is the breadth of the range.

The discrepancies between the results in the present study and those previously reported for *C. pallasi* (Dushkina, 1973) may also be in part due to experimental techniques. Fertilization assays conducted in the present study used sperm-egg ratios that yielded between 50% and 90% fertilization, rates that lie on the exponential, most sensitive, portion of a fertilization curve (Cherr *et al.*, 1990). The importance of utilizing such ratios is indicated by one of our experimental trials in which eggs were fertilized at sperm concentrations of 10^6 sperm/ml and 10^5 sperm/ml (Fig. 10). Although fertilization at the optimal salinity (16 ppt) differed by only 4%, the salinity tolerance curves for the two sperm concentrations were vastly different. At a sperm concentration of 10^5 sperm/ml, extremes at both ends of the salinity spectrum reduced fertilization to below 20%, whereas at a sperm concentration of 10^6

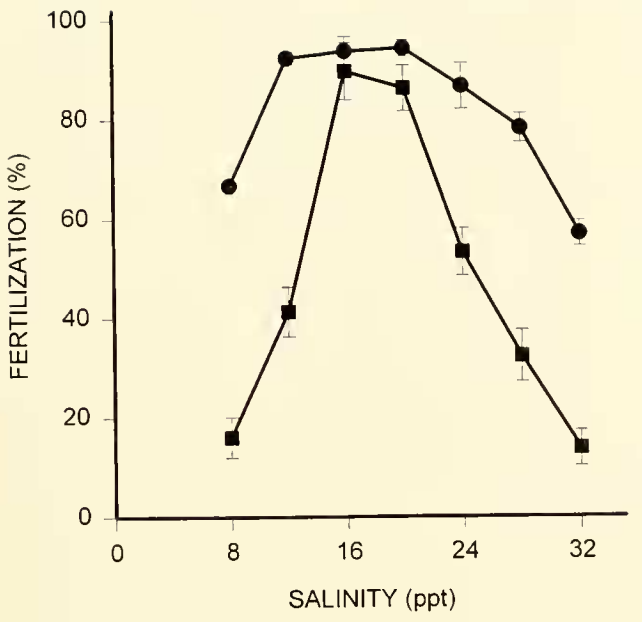

Figure 10. The effects of salinity on fertilization of *Clupea pallasi* eggs inseminated with 1×10^5 sperm/ml (■) and 1×10^6 sperm/ml (●); $n = 3$. Vertical lines = std. dev.

sperm/ml, fertilization remained above 50% even at 8 and 32 ppt salinity. This becomes very important in natural spawnings if the numbers of sperm reported at a British Columbia site (range of 100–150 sperm/ml) represent normal sperm concentrations in the wild (Hourston and Rosenthal, 1976).

Previous studies have shown that *C. pallasi* sperm maintain the ability to fertilize longer in lowered salinity water (*e.g.*, 10–15 ppt) than in full-strength seawater (Yanagimachi and Kanoh, 1953; Morisawa *et al.*, 1992). In the present study, fertilization reductions for *C. pallasi* at both high and low salinities correlated with the inhibition of sperm motility observed during *in vitro* assays with isolated SMIF (sperm motility initiation factor; Pillai *et al.*, 1993). Although the mechanism or mechanisms by which motility was inhibited were not investigated, three possibilities seem plausible: (1) The ability of SMIF to elicit motility could be affected; since the presence of SMIF in the micropyle area is essential for sperm motility (Yanagimachi *et al.*, 1992), its removal or inactivation would prevent fertilization. (2) External changes in Na^+, K^+, or both could perturb ionic movements across the sperm plasma membrane that are necessary for motility; support for this possibility comes from the current finding that fertilization is inhibited at elevated (≥300 m*M*) Na^+ in the presence of high (10 m*M*) K^+ and from a previous report that sperm become spontaneously motile (in the absence of SMIF) when placed in choline-substituted ASW that lacks Na^+ ions (Yanagimachi *et al.*, 1992). (3) There could be a general osmotic inhibition of motility at altered salinities; osmolality has been shown to affect sperm motility in a variety of freshwater and marine fish, but not in Pacific herring (Morisawa *et al.*, 1992). Osmolality alone was not sufficient to initiate sperm motility; only very low levels of motility were obtained when sperm were placed into hyperosmotic solutions of mannitol or even hyperosmotic solutions of NaCl and KCl (Morisawa *et al.*, 1992). Osmolality (under all three conditions) did influence the activity of sperm activating substance (now termed HSAP; Oda *et al.*, 1995) that was isolated from herring eggs (Morisawa *et al.*, 1992).

The effects of salinity on embryonic development in *C. pallasi* are not as absolute as those on fertilization. McMynn and Hoar (1953) reported that *C. pallasi* embryos possess a salinity tolerance range of 6–34 ppt, with an optimum at 11.5 ppt; however, the percent hatch achieved ranged from 0.2 to 11.8% in all salinities. Despite the inability of the study to decipher salinity effects on development, the authors reported two particularly sensitive stages of development—the completion of epiboly [called blastopore closure by McMynn and Hoar (1953)] and near the time of larval hatching (McMynn and Hoar, 1953). In the present study, there was no significant salinity effect through gastrulation (completion of epiboly). Effects of salinity were evidenced by (a) a delayed time to hatch and (b) a reduced number of larvae that successfully hatched. It is likely that these failures to complete development at both 3.5 and 28 ppt are manifestations of earlier developmental problems that we did not detect. It is also possible that energy reserves used to maintain ionic balances within the developing embryos at the extreme salinities depleted those needed for developmental progression, thus prolonging and ultimately halting development. Delay in the developmental program could lead to malformed embryos (*e.g.*, scoliotic larvae) or simply to larvae without the necessary energy reserves to survive hatching. Knowing whether the effects on development are the cumulative result of long-term or constant exposure to altered salinity or are a function of the developmental stage at which exposure occurs will be important in understanding the mechanisms by which salinity affects development.

Although we do not believe that salinity is the only factor governing successful reproduction of Pacific herring, it does appear to be an important component of both fertilization and embryonic development. Furthermore, comparisons of fertilization in the San Francisco Bay population with that of a Baltic population of *C. h. membras* and with published reports of other Pacific herring populations suggest that salinity tolerance ranges may differ between populations. The effects of salinity pressure on development may be more subtle than those on fertilization, yet may have more impact on recruitment in the

environment. Altered salinity not only reduces the number of normal, competent larvae that hatch, it also delays the hatching of those embryos. The costs of such delays, separations from larval schools, depleted energy reserves, and extended time in the chorion (where embryos are subject to both predation and exposure) influence chance for survival. A full understanding of the importance of salinity to herring reproduction will require knowledge of key physiological requirements for successful reproduction and recruitment and correlation of those requirements with environmental conditions in the bays and estuaries where defined populations of herring spawn.

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